BIOLOGICAL NITROGEN FIXATION BY EPIPHYTIC MICROORGANISMS IN RICE FIELDS[1]

ABSTRACT

Epiphytic microorganisms have been observed on shallow wetland rice, deepwater rice, and on weeds growing in rice fields. In the wetland rice field ecosystem, microorganisms epiphytic on rice and weeds make a limited contribution to the nitrogen input but blue-green algae play an important role providing an inoculum for the regeneration of the algal blooms that are periodically affected by adverse conditions. In deepwater rice, which offers a much greater biomass for colonization, the contribution of nitrogen by the epiphytic BGA is of agronomical significance. The measured activity corresponds to an input of about 10-20 kg N/ha per crop in the rice field mainly due to BGA.

Blue-green algae were found to grow preferentially on submerged decaying tissues. An endophytic growth inside the leaf sheath was also observed in deepwater rice. Observations support that algal epiphytism and endophytism are probably related to a mechanical effect rather than to biotic relationships.

[1]By S. A. Kulasooriya, postdoctoral fellow, University of Peradeniya, Sri Lanka; P. A. Roger, visiting scientist, Office de la Recherche Scientifique et Technique Outre-Mer, France; W. L. Barraquio, research assistant, and I. Watanabe, soil microbiologist, International Rice Research Institute, Los Baños, Laguna, Philippines. Submitted to the IRRI Research Paper Series Committee December 1979. The authors gratefully acknowledge the able assistance of Agnes Tirol, Wilbur Ventura who supervised the experiments in the deepwater tanks at IRRI, and Wisit Cholitkul who arranged their visit to the deepwater rice fields in Thailand. The study was partly supported by the United Nations Development Programme.

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It is well known that biological nitrogen fixation contributes significantly to the fertility of rice soils. Microorganisms operative in this process and their spatial distribution within a rice field ecosystem are illustrated in Figure 1. Studies on most of the components depicted in the figure have been recently reviewed: Dommergues and Rinaudo (1979) on the rhizosphere, Matsuguchi (1979) on heterotrophic bacteria, Roger and Reynaud (1979) and Venkataraman (1979) on the blue-green algae (BGA), and Watanabe (1978) and Becking (1979) on Azolla. There are a few reports on nitrogen-fixing bacteria associated with rice stems (Watanabe et al 1979, Watanabe and Barraquio 1979) and on nitrogen fixation by BGA epiphytic on freshwater macrophytes (Finke and Seeley 1978), but we are unaware of any studies on nitrogen fixation by epiphytic BGA in rice fields.

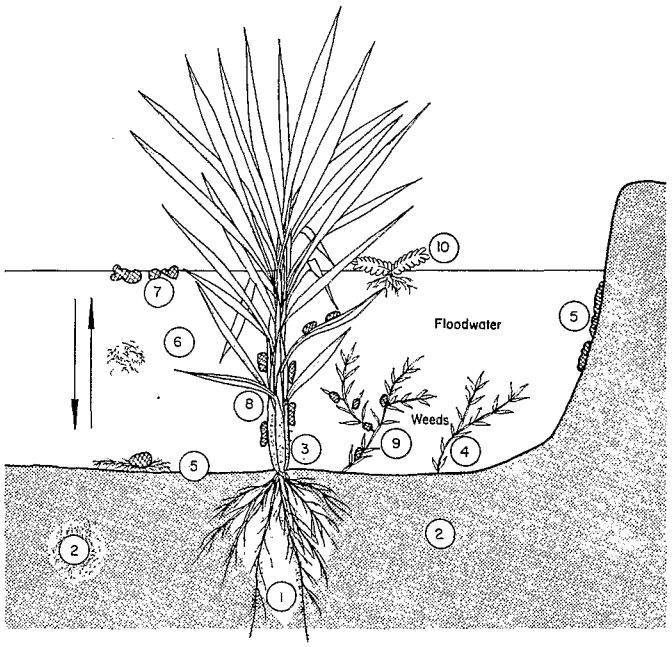

Fig. 1. Diagram of N_2-fixing components in a rice field ecosystem. Bacteria: (1) rhizosphere, (2) soil, (3) epiphytic on rice, (4) epiphytic on weeds. Cyanobacteria: (5) soil water interface, (6) free floating, (7) water air interface, (8) epiphytic on rice, (9) epiphytic on weeds. Azolla (10).

In rice fields, the epiphytic microflora appear to occupy an ecological niche with certain distinctive features. BGA, being attached in a somewhat permanent submerged position, are protected from dessication and inhibitory effects of high solar radiation (Reynaud and Roger 1979). This epiphytic habit is advantageous to the heterotrophic bacteria that may obtain nourishment from their hosts, but no nutritive association between the algae and the host plants is known.

In the wetland rice field ecosystem, epiphytism can occur on the submerged parts of rice plants and weeds. The submerged weed population can develop into a considerable biomass and in such cases the epiphytic microflora on them may make a significant contribution to the total nitrogen input. In deepwater rice, a large part of the plant remains under water and offers a substantial biomass for colonization by aquatic microorganisms. In addition, the submerged stems produce clusters of nodal roots that grow freely in the floodwater. Extracellular products from the epiphytic microflora on such roots may provide a source of nutrition to the rice plants. There are brief reports on the presence of nitrogen-fixing BGA on deepwater rice (Martinez and Catling 1978) and photodependent nitrogen-fixing activity associated with their nodal roots (IRRI 1977).

We studied epiphytic microorganisms and nitrogen-fixing activity associated with rice plants and weeds in wetland fields, and with deepwater rice, by microscopic examinations, algal and bacterial counts, and acetylene-reducing activity (ARA) measurements.

MATERIALS AND METHODS

Experimental method

An experiment was conducted in 1.5-m^2 wetland plots (5-8 cm water depth) with 5 treatments in triplicate, in a randomized block design. Each plot was planted separately to IR26 rice, submerged weeds (*Chara* sp. or *Najas* sp.), and nonsubmerged weeds (*Monochoria vaginalis* or *Cyperus iria*). Deepwater rice plants (DW6255) were grown in pots containing 7 kg (dry weight) of Maahas clay soil (Aquic Tropudalf) placed in a deepwater tank in which the water level was progressively increased 10 cm every other day to a final depth of 110 cm, which was maintained until crop maturity.

Sampling

Acetylene-reducing activity in the fields has been reported to exhibit a log-normal distribution (Roger et al 1977). Results of a study of the distribution law of epiphytic ARA among 35 rice hills (Fig. 2) confirmed that report and indicated a large variability of activities among the different hills. That implied that measurements should be made on replicates obtained from mixed material and not on a few randomly selected separate hills. Sampling

was, therefore, done from the complete harvest of a plot of IR26 and weeds and from seven plants for DW6255. For both rice varieties and nonsubmerged weeds, the root system and the aerial parts above the floodwater level were first excised, the remaining material mixed together, and random triplicate samples taken. With submerged weeds, the entire plant material was used for sampling. The samples were studied for their specific ARA and presence of epiphytic algae and bacteria.

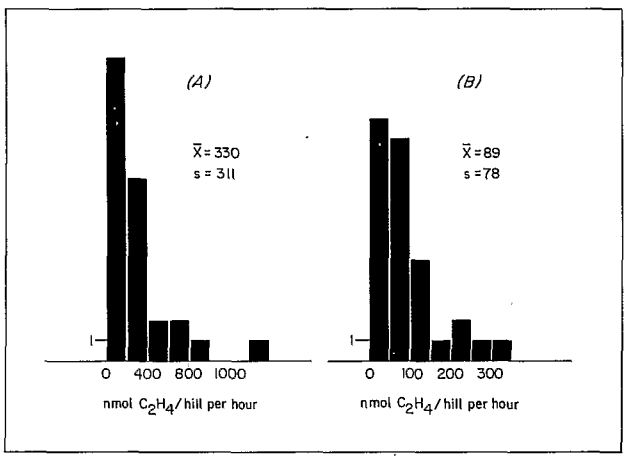

Fig. 2. Histograms showing the variations of: (A) light, (B) dark ARA among 35 hills from a plot at tillering stage.

Acetylene-reducing activity

ARA measurements were made in 250-ml Erlenmeyer flasks or in 900-ml plastic cylinders in an atmosphere of 10% acetylene in air. Incubation was either under 800 lx provided by fluorescent lights, or in the dark in aluminum-foil wrapped flasks. Plant material destined for dark incubation was covered in situ with a black cloth the day before harvesting, to eliminate any residual algal activity. Gas samples were removed after 0.5, 1, 2, 4, and 6 hours of incubation and analyzed by gas chromatography.

Algal counts

Algae were determined by plating macerated material on BG II medium (Allen and Stanier 1968) with and without combined nitrogen to estimate the total and the nitrogen-fixing algal populations, respectively. After incubating for 3 weeks at 30°C under continuous fluorescent light (800 lx), the plates were observed under a stereoscopic microscope, and algal colonies identified and counted.

Bacterial counts

Aerobic heterotrophic nitrogen-fixing bacteria were determined from the macerated material by the most probable number technique as described by Watanabe et al (1979). Inoculation was done in a semisolid glucose-yeast extract medium, which usually gives higher counts than malate medium (Watanabe et al 1979) and in malate-yeast extract medium to detect the presence of *Azospirillum* (Day and Döbereiner 1976).

Total aerobic heterotrophic bacteria were determined by spreading on tryptic-soy (0.1%) agar (1.5%) plates (Watanabe and Barraquio 1979). After incubating at 30°C for 1 week, the colonies were counted on the plates containing 30 to 300 colonies.

RESULTS AND DISCUSSION

Variation of epiphytism among rice hills

Light and dark ARA among 35 hills from the same plot are shown in Figures 2a and 2b. Both histograms exhibited a characteristic L shape. The standard deviation of the variables was very close to the mean. These features indicated a log-normal distribution of ARA in the light and in the dark. Similar results have been reported for ARA by soil algae and bacteria (Roger et al 1977).

Different kinds of epiphytism

Two types of algal epiphytism -- that visible to the naked eye, and that visible only under the microscope -- were observed. In the first type, globose gelatinous colonies of *Gloeotrichia* (2-10 mm diameter) were attached to the shallow wetland rice plants at the seedling and tillering stages, to *Chara* sp. filaments, and less frequently to deepwater rice. This growth was observed on both viable and necrosed host material. *Gloeotrichia* colonization was also observed on synthetic material such as old nylon strings. On the shallow wetland rice, *Gloeotrichia* epiphytism decreased between the seedling and tillering stages because algal masses detached from their hosts when gas bubbles formed within the colonies. Colonies attached to the living parts were observed to more easily dislodge than those attached to the necrosed parts.

The second epiphytic habit, seen only under the microscope, was predominantly due to *Nostoc*, *Calothrix*, and *Anabaena* sp., whose filaments grew firmly attached to the host surface. This was observed on submerged weeds, on shallow wetland rice at heading and maturity stages, and on deepwater rice.

Localization of epiphytism on the host

In the case of submerged weeds the distribution of *Gloeotrichia* colonies on the host was frequently unequal, with the older parts more heavily colonized. Even in microscopic epiphytism, colonization became progressively higher from the younger to the older parts of the host. This was quite apparent among the young, intermediate-age, and old leaves of *Najas*. These observations were confirmed by algal counts done separately on old and young parts of *Chara* sp.

and *Najas* sp. The total algal population on the old parts was four times that on the young parts.

Shallow wetland rice. Results of the comparison of epiphytism on the most outer (outer) and the other (inner) leaf sheaths of shallow wetland rice (Table 1) indicated that both ARA and microbial colonization on the outer sheaths were much higher than on the inner sheaths, irrespective of the type of microorganisms. Experiments using ^{15}N-labeled N_2 have also shown a higher nitrogen-fixing activity on the outer leaf sheaths than on the inner ones (Ito et al 1980).

In the case of algae the difference in nitrogen-fixing activity may be related to light availability. Nitrogen-fixing algae present on the inner sheaths (5.3×10^3/g fresh weight) were mainly spores or inactive forms as demonstrated by the negligible difference between dark and light ARA measurements on the inner sheaths (Table 1). The much higher density of bacteria on the outer sheaths may indicate that outer parts contain partially decomposing material, which provides suitable substrates for bacterial growth.

Deepwater rice. Deepwater rice plants at maturity exhibited vertical growth of the lower part in the water (submerged) followed by horizontal growth of the upper part just under the water surface (floating), from which aerial tillers grew upward (Fig. 3).

Microscopic examinations for epiphytic algae showed the presence of BGA, green algae, and diatoms attached to the surface of exposed roots, leaf sheaths, inner roots (enclosed by leaf sheath), and culm. The predominant BGA were nitrogen-fixing types, notably *Nostoc*, *Anabaena*, *Calothrix*, and *Gloeotrichia*. With regard to the distribution of these epiphytic species, no differences were observed between the upper and the lower plant parts. However, *Nostoc* colonies were frequently present at the points of lateral branching of roots, as already reported by Watanabe (IRRI 1977), whereas *Calothrix* did not show any such preference. *Gloeotrichia* was more common on the decaying leaves and leaf sheaths than on other parts. Observations made on dissected parts and sections indicated that:

- Species of *Nostoc* and *Calothrix* were present inside the leaf sheaths.
- The algae were present inside the cavities of the leaf sheaths but not within the host cells.
- This *endophytism* was common among senescent or dead material but absent in living tissues.

Microscopic examination revealed an unequal distribution of epiphytic algae among the different plant organs -- exposed roots, leaf sheaths, inner roots, and culm portions (Fig. 4). Because of that we separately used aliquot samples of those for ARA and microbial enumerations.

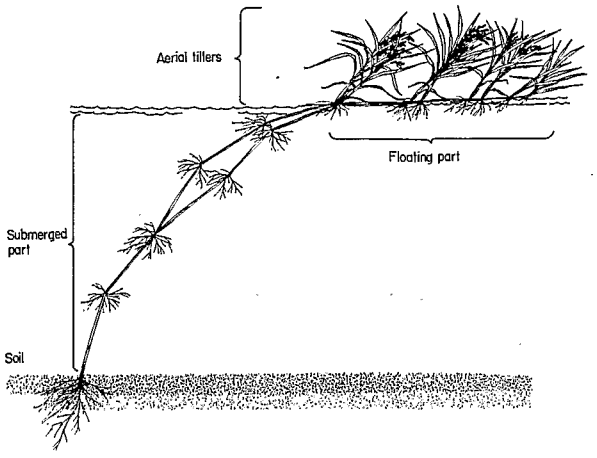

Fig. 3. Diagram of a deepwater rice plant at maturity.

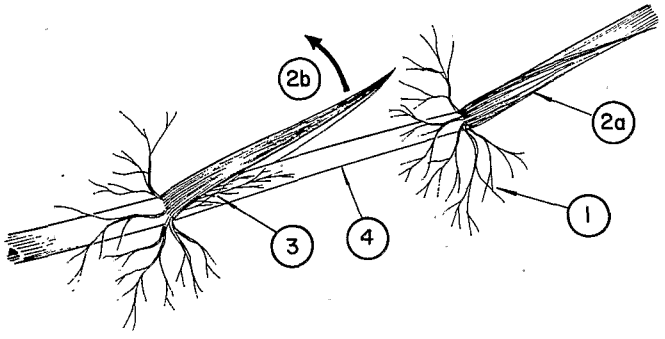

Fig. 4. Component parts of deepwater rice available for epiphytism. 1) exposed roots; 2a) leaf sheath in position; 2b) leaf sheath opened out; 3) inner roots; and 4) culm.

Results of microbial observations and ARA measurements on the component parts are presented in Tables 2 and 3. Within the limits imposed by accuracy of the method (Roger and Reynaud 1978), no significant differences between microorganisms enumerated on submerged and floating parts were observed. Among the different components of the plants, the culm supported the lowest number of microorganisms (Table 2).

The ARA measurements (Table 3) clearly show that ARA in the light was always higher than that in the dark and that the specific ARA associated with the floating parts were higher than the corresponding ARA on the submerged parts. Of the different plant components, the inner roots on the floating parts exhibited the highest specific activity, followed by leaf sheath, exposed roots, and culm. However, the total ARA of each part (specific ARA x fresh weight) was highest on the leaf sheath, followed by the culm and exposed roots.

Relative contribution of microorganisms

Counts indicated the presence of both nitrogen-fixing BGA and bacteria on the different hosts studied (Table 4). Bacterial counts on tryptic-soy agar revealed high populations of total aerobic heterotrophs. Compared to the heterotrophs, nitrogen-fixing populations were low. Growth on glucose medium showed the presence of acid-gas-producing organisms (probably Enterobacteriaceae). Growth on malate revealed *Azospirillum*-like organisms. The presence of *Azospirillum*-like bacteria on rice has been reported by Watanabe et al (1979).

BGA counts indicated the presence of *Gloeotrichia*, *Nostoc*, *Calothrix*, and *Anabaena* as nitrogen-fixing epiphytic types. Shallow wetland rice, deepwater rice, and *Chara* sp. had similar algal densities. *Najas* had a lower density.

ARA measurements in the light and in the dark (Table 5) indicated that heterotrophic nitrogen fixation was relatively constant and low (1-10 nmol/g per hour), compared to the phototrophic activity (1-600 nmol/g per hour). The relative contribution of heterotrophic bacteria was high only when the algal nitrogen-fixing activity became low.

However, counts on selective media gave high densities of epiphytic nitrogen-fixing bacteria (Table 4), but dark ARA measurements did not yield results compatible with such populations. Because all the ARA measurements were done in air, it is possible that a substantial part of the activity due to microaerophilic organisms could have been inhibited. The presence of such microaerobic sites in situ is likely, considering the large populations of heterotrophic bacteria (Table 4) associated with the decaying host material.

Variations of epiphytism and ARA along the cultivation cycle

A remarkable change was observed in the algal epiphytism along the developmental cycle of the shallow wetland rice plant, with a corresponding change in the light ARA.

Table 1. Distribution of ARA (nmol C_2H_4/g fresh wt per hour) and epiphytic microorganisms (no./g fresh wt of host material) between outer and inner parts of rice sheath at heading stage.

		Outer sheath	Inner sheath
ARA	Light	2.5	0.15
	Dark	0.5	0.11
Total algal flora		3.5×10^5	1.7×10^4
Nitrogen-fixing algae		1.2×10^5	5.3×10^3
Total aerobic heterotrophs		4.7×10^8	3.0×10^6
Nitrogen fixers on glucose (Enterobacteriaceae)		2.0×10^7	9.5×10^4
Nitrogen fixers on malate (*Azospirillum*-like)		9.5×10^6	9.5×10^3

Table 2. Epiphytic microorganisms counted from deepwater rice at maturity.

Component parts of plants	Epiphytic N_2-fixing BGA (no./g fresh wt x 10^{-4})	Epiphytic bacteria (no./g fresh wt x 10^{-6})		
		Total heterotrophs	N_2 fixers (Glucose)[a]	N_2 fixers Malate[b]
Floating				
Exposed roots	128	1180	139	11
Leaf sheath	68	1360	32	32
Inner roots[c]	n.d.	n.d.	n.d.	n.d.
Culm	4	94	3	3
Submerged				
Exposed roots	64	1300	14	27
Leaf sheath	150	2400	44	44
Inner roots	40	1340	6	26
Culm	5	300	27	27

[a]Enterobacteriaceae. [b]*Azospirillum*-like. [c]n.d. = not determined.

Epiphytic nitrogen-fixing activity was primarily due to a visible growth of *Gloeotrichia*, which was predominant during the early stages of rice growth. At the seedling stage, the rice stems had an epiphytic *Gloeotrichia* biomass of about 2 t/ha (fresh weight) and ARA in the light was 51 µmol C_2H_4/m^2 per hour. At tillering, this biomass diminished to 0.5 t/ha with an activity of 15 µmol C_2H_4/m^2 per hour. The epiphytic algae exhibited the same specific activity at these two stages (about 2.4 nmol C_2H_4/mg prot. per minute. Therefore, the ARA decrease observed from seedling to tillering thereafter was due to a decrease of the biomass of epiphytic *Gloeotrichia* that was detached from their host and floated. At heading and maturity, algal epiphytism was not visible and the light ARA had decreased to low values: 1.2 and 2.5 µmol C_2H_4/m^2 per hour, respectively. Nevertheless, counts on the rice stems showed the presence of several epiphytic nitrogen-fixing algae with *Nostoc* and *Calothrix* as dominant species. These results show that the algae, although present during these stages, probably existed to a large extent as quiescent cells or propagules and contributed little nitrogen to the crop. This decrease in algal biomass and its activity was possibly related to a dramatic decrease of light availability at the start of the rainy season and an increase of the rice canopy.

Along the cultivation cycle, dark ARA remained low (0.3 to 2.5 µmol C_2H_4/m^2 per hour) and relatively unchanged from tillering to maturity. Range of dark ARA on rice stems (bacterial activity) was in agreement with the results reported by Watanabe et al (1979).

In deepwater rice no macroscopic epiphytism by *Gloeotrichia* was observed and both microscopic examinations and counts indicated no changes of the

Table 3. Acetylene-reducing activity on the component parts of deepwater rice at maturity.

Component parts of plant	Biomass (g fresh wt per plant)	Light		Dark	
		Specific ARA nmol C_2H_4/g (fresh wt) per h)	ARA[a] nmol C_2H_4 per h	Specific ARA nmol C_2H_4/g (fresh wt) per h)	ARA[a] nmol C_2H_4/h
Floating					
Exposed roots	2.5	24	61	0.06	0.15
Leaf sheath	62	54	3348	1.5	93
Inner roots	0.25	69	17	0.03	0.01
Culm	98	2.5	245	0.5	49
Submerged					
Exposed roots	64.3	0.54	35	0.08	5
Leaf sheath	15	12	180	0.1	1.5
Inner roots	1.3	3	4	0.05	0.06
Culm	100	0.3	30	-0.02	2

[a] Specific ARA x component biomass.

Table 4. Numbers of epiphytic microorganisms counted.

Host	N_2-fixing BGA per g (fresh wt) of host	Bacteria per g fresh wt of host[a]		
		Heterotrophs	N_2 fixers (Glucose)	N_2 fixers (Malate)
Shallow wetland rice (heading)	4.8×10^5	1.8×10^8	7.5×10^5	3.7×10^6
Deepwater rice (heading)	2.3×10^5	n.d.	n.d.	n.d.
Deepwater rice (maturity)	3.5×10^5	7.2×10^8	2.0×10^7	2.1×10^7
Chara sp.	4.8×10^5	1.7×10^8	4.3×10^5	3.2×10^5
Najas sp.	8.5×10^4	6.5×10^7	5.5×10^4	1.8×10^5

[a] n.d. = not determined.

Table 5. Specific ARA measurement on rice and weeds.

Sample	Dominant N_2-fixing BGA	ARA (nmol C_2H_4/g fresh wt per h)		Dark ARA as a % of the light ARA
		Light	Dark	
Rice at seedling stage	*Gloeotrichia*	614.0	n.d.	n.d.
Rice at tillering stage	*Gloeotrichia*	37.5	10.1	27
Rice at heading stage	*Nostoc*	1.9	0.67	25
Rice at maturity	*Calothrix*	1.1	1.1	100
Deepwater rice at heading	*Nostoc* *Calothrix* *Anabaena*	15.9	4.8	30
Deepwater rice at maturity	*Nostoc* *Calothrix* *Anabaena*	9.7	1.0	10
Chara	*Gloeotrichia* *Nostoc* *Calothrix*	36.6	3.3	9
Najas	*Nostoc* *Calothrix*	26.7	1.8	7
Monochoria	*Nostoc* *Calothrix*	1.8	1.3	72
Cyperus	*Nostoc* *Calothrix*	4.4	2.5	57

epiphytic algae along the cultivation cycle. The dramatic decrease in light ARA observed with shallow wetland rice toward the end of the cultivation cycle was also not encountered with deepwater rice. This was possibly due to the morphology of the deepwater rice plant, which provided a less dense canopy than the shallow wetland rice.

The decrease of the specific activity from heading (20 nmol C_2H_4/g fresh weight per hour) to maturity (10 nmol C_2H_4/g per hour) was compensated by an increase of the host biomass so that a constant activity of 1.5 mmol C_2H_4/m^2 per day was measured at both stages, on the basis of 25 plants/m^2 and a 12:12 hour day/night cycle (Table 6).

Table 6. Acetylene-reducing activity on deepwater rice.

	Stage of plant growth	
	Heading	Maturity
Specific ARA nmol C_2H_4/g fresh wt host per hour	20.7	10.7
Host biomass gram	246	478
ARA per plant µmol C_2H_4/ hour	5.1	5.1
Extrapolated to field mmol C_2H_4/m^2 per day	1.5	1.5

Nature of the association

The results we obtained are insufficient to fully explain the relationships between the algal epiphytes and their hosts but certain inferences can be drawn.

Gloeotrichia has been reported to be epiphytic on aquatic plants (Fremy 1930, Finke and Seeley 1978). Our experience shows that it does not exhibit any selectivity between dead and living, organic or inorganic material, but seems to grow preferentially on rough surfaces as indicated by the following observations:

- Epiphytism on *Chara* sp., which has a rough corticated surface, was more than on *Najas* sp.
- Colonies on living, smooth rice stems detached more easily than those on dead plant material, which as demonstrated by Howard-Williams et al (1978), has rough surfaces.
- Colonization was observed on old, rough nylon strings but not on new smooth ones placed in the floodwater. Similar colonization on polyethylene strips has been reported by Finke and Seeley (1978).

In the case of *microscopic epiphytism* most of the isolated epiphytic strains grew appressed to the surface of the culture vessels and rarely formed floating colonies.

A unique finding of this study was the presence of BGA inside the leaf sheaths of deepwater rice. This finding on rice plants grown in experimental plots at IRRI was confirmed by microscopic examinations on samples collected from deepwater rice fields in Nakorn Nayok (Thailand), which showed a high density of a true-branching, heterocystous BGA within the leaf sheaths. Endophytic algae were present in senescent and necrosed material but not in living tissues. This phenomenon was not specific to deepwater rice, and the same was observed in the decaying leaf sheaths of associated grasses. The results obtained do not confirm the existence or absence of biotic relationships between the algae and their hosts, but indicate that a mechanical effect in relation to the roughness of the support is involved in algal epiphytism and endophytism. The roughness of the host surface can be a characteristic of the plant (corticated filaments of *Chara* sp.) or the result of decay.

Agronomic significance of epiphytism

From the ARA measurements of these experiments the nitrogen input in the rice field ecosystem by organisms epiphytic on shallow wetland rice can be evaluated to a few (2-3) kilograms per hectare per crop, mainly due to the activity of *Gloeotrichia*.

Among the different weeds studied, only the submerged ones exhibited a non-negligible activity approximately corresponding to an input of 2 kg N/ha per crop under rice and 4 kg N/ha under fallow.

The activity measured on deepwater rice corresponds to an input of about 10-20 kg N/ha per crop in the rice field. This substantial contribution was mainly due to the greater biomass available for colonization rather than heavier colonization by epiphytes.

The ARA rates obtained in this study were measured under low light intensity and aerobic conditions using cut material removed from the field. These values, therefore, should be considered an underestimation of the in situ activity.

Another important role of algal epiphytism is the regeneration of nitrogen-fixing algal blooms, which are frequently washed out of the field by heavy rains or bleached by high light intensities. Epiphytic algae are protected from the adverse conditions and provide an inoculum from which regeneration of the bloom is possible.

CONCLUSION

We conclude that, in the shallow wetland rice field ecosystem, epiphytism by nitrogen-fixing BGA makes only a small contribution to the nitrogen input, but plays an important role in inoculum conservation. On the other hand, epiphytic algae on deepwater rice produce a substantial nitrogen input.

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S. A. KULASOORIYA, P. A. ROGER,
W. L. BARRAQUIO, and I. WATANABE

The International Rice Research Institute
P.O. Box 933, Manila, Philippines